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Full Length Research Paper

Molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in province of Najaf, Iraq

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ABSTRACT

Avian influenza virus subtype 9 and Newcastle virus have been recognized as the most important pathogens in poultry. In Najaf governorate, Iraq, many chickens flocks suffered from high mortality (about 30-70%) with respiratory signs. In this study, trachea swabs and tissue specimens from 53 commercial broiler chicken flocks suffering from respiratory diseases were tested initially by rapid test for antigen influenza type A and by reverse transcription PCR for avian flu virus type A, H5, H7, H9, and Newcastle disease virus (NDV).

The reverse transcription PCR results showed that 75% of these flocks were infected with both avian flu virus type H9 and NDV whereas 25% of them were infected only by H9. On the other hand all flocks were negative for subtypes H5, H7. Our data showed that above mentioned respiratory pathogens were the most important agents of respiratory diseases giving high mortality in broiler chicken in the area of the study.

Key words: Respiratory Diseases, Avian flu, broiler chicken, Molecular Diagnosis, Iraq.

Introduction

Viruses and bacteria cause respiratory diseases or interact to cause disease (Yashpal *et al*., 2004). Bird flu is an A type virus with multiple subtypes, these being defined by combinations of two proteins. These two proteins (HA and NA) are exist on the surface of the virus. The HA protein has 16 different subtypes while the NA has nine subtypes. The combination formed by one HA and one NA protein is used to name the virus subtype (CDC, 2006). Bird flu is known as H5N1 virus, being a combination of HA 5 and NA 1 proteins. There are also H7 and H9 types of bird flu.

Avian Influenza viruses are also classified by their level of pathogenicity, or virulence. Highly pathogenic avian influenza (HPAI) has a high mortality rate in poultry; capable of killing between 90 and 100 percent of infected chickens (Alexander, 2000). Low pathogenic avian influenza (LPAI) causes less severe symptom. Newcastle disease (ND) is a highly contagious viral disease that attacks many species of domestic and wild birds (Al-Garib *et al*., 2003). The causal agent is the Newcastle disease virus (NDV) which is a negative-sense single-stranded RNA virus belonging to the family *Paramyxoviridae*. Through restriction site mapping and sequence analysis of the fusion gene (F-gene), NDV strains have been divided into eight genotypes (Ballagi *et al*., 1996). The strains are also classified into highly virulent (velogenic), intermediate (mesogenic) or a virulent (lentogenic) based on their pathogenicity in chickens (Beard and Hanson,1984).

Many endemic respiratory infectious diseases in the province of Najaf, Iraq continue to decrease the profitability of commercial poultry production. Flocks in Najaf government suffered from these diseases which causes high morbidity, mortality, and respiratory signs. The etiology of respiratory diseases are complex often involving more than one pathogen at the same time (Yashpal, *et al.,* 2004) including avian influenza virus subtype H9 and Newcastle virus. These pathogens are of major important because they can cause disease independently, or in association with bacteria (Roussan *et al*., 2008).

The RT-PCR technique can detected avian influenza type A screening all flocks can appeared positive. Subtype H9 can detected all flocks too. 75% mixing infected H9 with NDV and all broiler chicken flocks free from H5, H7.25% of flocks infected only H9.

The incidence and severity of respiratory disease in commercial broiler chicken flocks have increased recently in Iraq because of intensification of the broiler industry. Because of health and economic damage caused by avian Influenza viruses, this study was conducted in province of Najaf, Iraq.

Material and Methods

During the period from June to December 2008, the avian flu virus, stroke the commercial broiler chicken flocks in province of Najaf, Iraq causing high mortality and sever clinical signs. Broiler chicken vaccination program usually involved oily vaccine ND injection and IB spray at 1 day old. In the seven day, they vaccinated Gumboro mild. In the tenth day, they vaccinated NDV by drinking water. In14th day they vaccinated moderate Gumboro. On another hand, some of broiler chicken they vaccinated program 3 attenuated live ND by drinking water in the 7, 18, 30 days of age and vaccinated IBD mild strain in 7day and moderate in 14 day of their age. None of these flocks were vaccinated against avian flu subtype H9. In these flocks respiratory diseases appeared at 20th day of age or above perhaps in 25th day of age. The main pathological or clinical signs, included depression, rhinitis, gasping coughing, conjunctivitis, ocular discharge, weakness, and diarrhea.

The cross lesion appeared severe congestion of trachea with mucopurelant exudates, air securities and perihepatitis and pericarditis, in addition to intestinal congestion.

Tracheal swabs and tissue specimens were collected from positive (infected) cases and avian flu type A antigen (anigen corp) was detected using rapid test kit. Reverse transcription PCR was used for detection of avian flu type A subtypes H9, H7, H5, NDV.

RNA extraction

Tracheal swab and tissue specimens from lung were collected from each flock. Tracheal swabs were placed in 1.0 ml of normal saline and centrifuged at 1000 g/min for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 100 µL (obtained from sacace corp).

For preparation of tissue specimen, 1gm (trachea ,lung) homogenized with mechanical homogenizer was dissolved in 1.0 ml of normal saline ,vortexed vigorously and incubated for 30 minutes at room temperature, then the supernatant was transferred into a new 1.5 ml tube (obtained from sacace corp).

Extraction of RNA was performed on 100 µL of prepared sample from each flock for purification of RNA, according to the manufacturer procedure (sacace biotechnologies).

RT-PCR (reverse transcription PCR) the avian flu type A and subtypes H7,H5 virus were detected by two steps RT-PCR was performed by using a RT-PCR system kit (sacace corp) according to the manufacturer instructions (sacace biotechnologies). NDV and subtype H9 viruses were detected using one step RT-PCR and performed by RT-PCR system kit (antigen corp) according to the manufacturer instructions.

For each flock, 5 PCR tubes were prepared. The first tube was a screening for avian flu type A virus which was amplified using the fallowing conditions in DNA engine thermal cycler (PCR system, obtained from Singapore). In the first step, RT-PCR was carried out for reverse transcription cycle of 30 minutes at 37 ºC. The second step included putting the cDNA with amplification kit into the thermal cycler according to the following programme: 95 ºC for 5 min, then 42 PCR cycle at 95 ºC for 1min (denaturation. 63 ºC for 1min (annealing) 72 ºC for 1min (extension) with final extension cycle at 72 ºC for 1min (sacace corp), when the sample appeared positive. A another 4 PCR tubes for subtypes H7, H5, H9, and NDV were made. The steps for amplification of subtypes H7, H5 was the same but with different annealing temperatures, H5 (58 ºC) and H7 (61 ºC).

For subtypes H9 and NDV, a different programme was followed, as one step RT-PCR was performed using an additional RT-PCR system kit (anigen corp) according to the manufacturer instructions. Samples of PCR for H9 and NDV were amplified using the following conditions and were carried out in the same run. RT-PCR was carried out for one reverse transcription cycle for 30 min at 42 ºC followed by 94 ºC for 15 min, then 40 PCR cycles at 94 ºC for 30 sec (denaturation), 50 ºC for 30 sec (annealing) and 72 ºC for 40 sec (extension), with final extensions cycle at 72 ºC for 5 min. Positive and negative control for RNA extraction and RT-PCR were used in each run.

Agarose gel electrophoresis:

PCR products were electrophored on 2% agarose gel in TBE buffer (40 mM of tris and 2 mM of EDTA, with PH value of 8.0) containing ethidium bromide for 20 min at 110 volt for type A, H7,H5 (sacace corp), while type H9 and NDV were electrophoresed on 1.5 % agarose gel in TBE buffer containing ethidium bromide at 110 volt for 20 minutes (anigen corp.). Magnified products were visualized under UV light.

Results:

The 53 commercial broiler flocks with history of respiratory signs and high mortality in Najaf governorate, Iraq were positive for rapid influenza type A antigen. Results of RT-PCR revealed that 53 (100%) of the flocks were infected by avian type A virus (Figure-1). Of which, 40 (75%) were infected with both H9 and NDV viruses, while 13 flocks 25% were infected only by H9 virus (Figures 2,3). However, all of these flocks (100%) were negative for H5, H7 viruses (Figure-4).

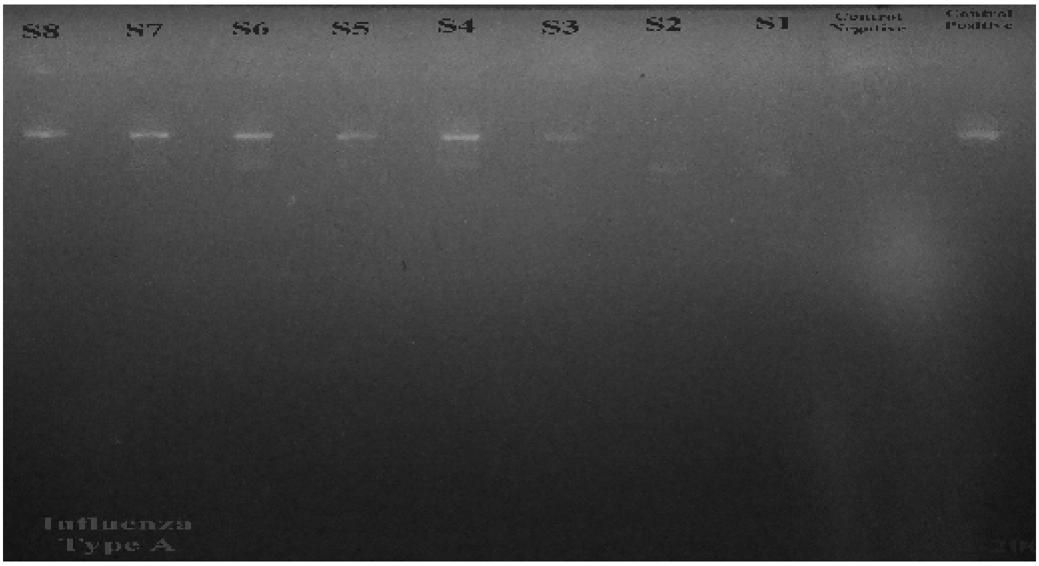


Figure-1: Electrophoresis analysis for avian flu type A virus. Samples 4,5,6,7,8 are positive. Control band 270 bp.

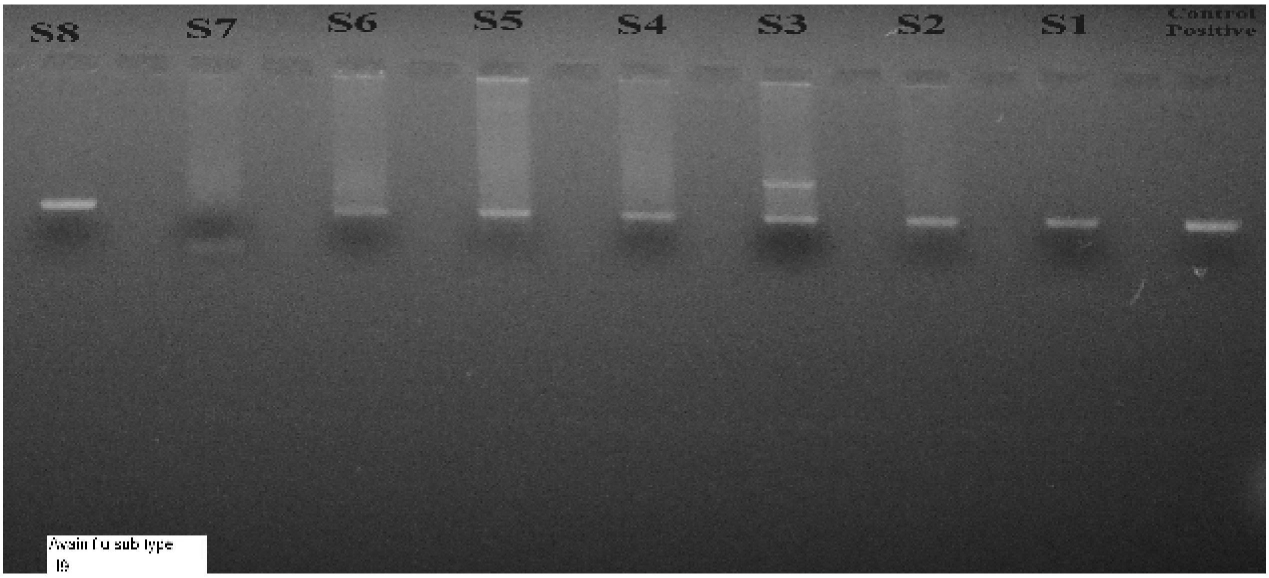


Figure-2: Electrophoresis analysis for avian flu subtype H9. Samples 1,2,3,4,5,6,7,8 are positive samples. Control band 230 pb.

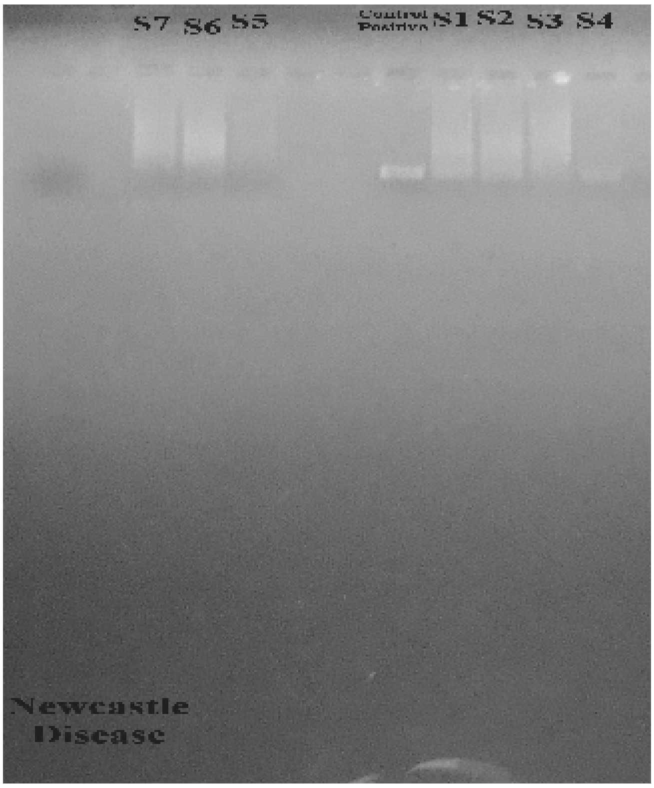


Figure-3: Electrophoresis analysis for ND virus. Control band 204 bp, Samples 1,2,3,4,6,7 are positive. Sample 5 is negative.



Figure-4: Electrophoresis analysis for avian flu Subtype H7. Positive control band 360 bp . All samples are negative.

Discussion:

Respiratory diseases in poultry have been reported to be as mixed or single cause of infections with several agents (Yashpal *et al.*, 2004). The avian influenza virus subtype H9 and Newcastle disease virus are the major cause of a respiratory tract infection of broiler chickens and every year bring about high morbidity and mortality in flocks in province of Najaf, Iraq. Clinically it is impossible to distinguish avian influenza virus infection of upper respiratory tract from Newcastle disease, so RT-PCR is used as essential tool for diagnosis of these diseases.

Results of RT-PCR in this study revealed that all the flocks (53) were infected with avian influenza virus H9 virus. Forty of them were mixed infections with both H9 and NDV while 13 flocks diagnosed only H9 subtype. All flocks were non-infected by any of H7, H5 subtypes. These results suggested that H9 and NDV were the most important causes of avian respiratory diseases and they are responsible for the high mortality and respiratory signs in broiler chicken in the area of the study. It is common practices in Najaf government, to vaccinate broiler flocks against H9 especially against local strains to control this virus and improve the management of the fields.

Avian flu virus subtype H9 has been reported in commercial chicken in different countries in Asia, Pakistan (Naeem *et al*., 1999), Iran (Nilli and Asasi, 2003), United Arab Emirate (Manvell *et al*., 2000), Saudi Arabia (Banks *et al*., 2000), Korea (Kwon *et al*., 2006), and Jordan (Roussan *et. al.,* 2008).

Despite the use of NDV vaccine, Its common to find ND infection in vaccinated broiler flocks especially after exposure of the fields to infection with H9 virus which expressed to co-infection with NDV. This study recommended that the vaccination against ND should not be hold until the titer of these viruses detected by HI (hemagglutination inhibition) or ELISA test, in the field, be decreased.

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